Cancer–Testis Antigen Expression in Digestive Tract Carcinomas: Frequent Expression in Esophageal Squamous Cell Carcinoma and Its Precursor Lesions

Yao-Tseng Chen1,2, Nicole C. Panarelli1, Kathryn C. Piotti1, and Rhonda K. Yantiss1

Abstract

Cancer–testis (CT) antigens are attractive tumor antigens for cancer immunotherapy. They comprise a group of proteins normally expressed in germ cells and aberrantly activated in a variety of human cancers. The protein expression of eight cancer–testis antigens [MAGEA, NY-ESO-1, GAGE, MAGEC1 (CT7), MAGEC2 (CT10), CT45, SAGE1, and NXF2] was evaluated by immunohistochemistry in 61 esophageal carcinomas (40 adenocarcinoma and 21 squamous cell carcinoma), 50 gastric carcinomas (34 diffuse and 16 intestinal type), and 141 colorectal carcinomas. The highest frequency of expression was found in esophageal squamous cell carcinomas. Positive staining for MAGEA, CT45, CT7, SAGE1, GAGE, NXF2, NY-ESO-1, and CT10 was observed in 57%, 38%, 33%, 33%, 29%, 29%, 19%, and 14% of squamous cell carcinomas, respectively. Similar staining patterns were observed in squamous dysplasias. Expression frequencies of cancer–testis antigens were seen in 2% to 24% of gastroesophageal adenocarcinomas and were not significantly different between adenocarcinomas of the stomach versus the esophagus, or between diffuse and intestinal types of gastric adenocarcinomas. Colorectal cancers did not express NY-ESO-1, CT7, CT10, or GAGE, and only infrequently expressed SAGE1 (0.7%) MAGEA (1.4%), CT45 (3.5%), and NXF2 (8.5%). We conclude that cancer–testis antigens are frequently expressed in esophageal squamous neoplasms. Although cancer–testis antigens are generally considered to be expressed later in tumor progression, they are found in squamous dysplasias, suggesting a potential diagnostic role for cancer–testis antigens in the evaluation of premalignant squamous lesions. Cancer Immunol Res; 1–7. ©2013 AACR.

Introduction

Cancer–testis (CT) antigens are protein antigens that are normally expressed in the premeiotic germ cells of adult testis and in developing fetal testis and ovary, but not in any other adult tissues. Analysis of various types of human cancer has shown cancer–testis gene activation and protein expression in a proportion of human cancers in a lineage-unrelated fashion (1–4) and cancer–testis antigens can be considered as tumor–specific antigens in these tumors. Of more than 100 cancer–testis antigens identified, approximately 30 are encoded by multigene families on chromosome X (CT-X genes), including MAGEA, NY-ESO-1, GAGE, CT7, CT10, CT45, SAGE1, and NXF2 (2, 5–13). The tumor specificity of the CT-X antigens, in conjunction with their capability to elicit humoral and cellular immune responses in patients with cancer, led to the recognition of these antigens as promising therapeutic cancer vaccine targets. Cancer vaccine trials using CT-X antigens, MAGEA3 and NY-ESO-1 in particular, are ongoing and promising preliminary data have been reported (14–16).

This diagnostic and therapeutic potential of CT-X antigen is tumor type–dependent as the frequency of CT-X antigen expression is highly variable among different tumor types. Melanoma, ovarian cancer, lung cancer, and bladder cancer are examples of “CT-rich” tumors, whereas renal cancer, colorectal cancer, and most lymphoma and leukemia—except adult T-cell leukemia/lymphoma (17) and multiple myeloma (18)—are “CT-poor,” rarely expressing CT-X antigens (4, 12). Different histologic cancer types, even within the same organ system, show variable frequencies of CT-X expression. Squamous cell carcinoma in the lung, for example, expresses CT-X at frequencies significantly higher than adenocarcinoma (4, 6). Furthermore, for a given histologic type, expression of CT-X genes in cancer has been correlated to tumor progression and more aggressive clinical behavior (4). In urothelial carcinoma, expression of MAGEA and NY-ESO-1 was more often found in high-grade carcinomas in comparison with low-grade cancers (19, 20). In breast carcinoma, the expression of multiple CT-X antigens was more frequent in hormone-receptor–negative carcinomas and in tumors greater than 2 cm in size (21, 22).

Several studies have examined the expression of CT-X antigens in carcinomas of the digestive tract, either at the mRNA level by RT-PCR (23–26) or, more recently, at the protein level by immunohistochemical (IHC) analysis (25, 27–30). The latter is clearly more relevant clinically, either as a diagnostic marker...
or in predicting patients that might benefit from cancer–testis antigen–based cancer vaccines, as posttranscriptional control of gene expression can lead to substantial discordance between mRNA and protein expression in cancer cells. The analysis of cancer–testis protein expression has been limited by the availability of antibody reagents against these antigens. Most studies evaluating cancer–testis antigen immunohistochemistry have limited their scope to the evaluation of MAGEA and NY-ESO-1 with rare exception (27). To evaluate the potential of cancer–testis antigens in the immunotherapy for digestive tract carcinomas, the protein expression frequency of eight CT-X antigens (MAGEA, NY-ESO-1, GAGE, CT7, CT10, CT45, SAGE1, and NXF2) in esophageal, gastric, and colorectal carcinomas was determined using a panel of anti-CT-X antibodies, including a few generated in our laboratory.

Materials and Methods

**Tissues and tissue microarrays**

Formalin-fixed paraffin-embedded tissues used for this study were procured from the Department of Pathology and Laboratory Medicine at New York Presbyterian Hospital-Weill Cornell Medical Center (New York, NY) following protocols approved by the Institutional Review Board (IRB). Surgical pathology reports from 2000 to 2010 were searched for resected esophageal, gastric, and colorectal carcinoma, and 61 esophageal carcinomas (40 adenocarcinoma and 21 squamous cell carcinoma), 50 gastric carcinomas (34 diffuse and 16 intestinal types), and 141 colorectal carcinomas (40 adenocarcinoma and 21 squamous cell carcinoma) were used to construct tissue microarrays (TMA) for this study. Each case was represented by three 0.6-mm tissue cores. To evaluate cancer–testis expression in early squamous neoplasms, 22 esophageal biopsy specimens with histologic diagnoses of squamous dysplasia or carcinoma, and 22 esophageal biopsy specimens were evaluated for CT-X protein expression using whole sections.

**Monoclonal and polyclonal antibodies**

The antibodies used are summarized in Table 1. Antibodies against MAGEA, GAGE, and SAGE1 were purchased commercially. Of these, the GAGE antibody is expected to react with all GAGE gene products due to the extremely high-sequence homology among the GAGE proteins, including GAGE-7, the protein against which the antibody was produced. MAGEA monoclonal antibody 6C1 is produced against MAGEA1 and is broadly reactive for gene products of the MAGEA multigene family, including MAGEA1, A2, A3, A4, A6, A10, and A12 proteins (31). Antibodies against cancer–testis antigens NY-ESO-1, CT7, CT10, CT45, and NXF2 were produced and characterized in our laboratory and have been previously described (32). Nuclear staining for CT10, CT45, SAGE1, and NXF2 was considered a positive result, whereas nuclear and/or cytoplasmic staining of MAGEA, NY-ESO-1, CT7, and GAGE were interpreted to be positive staining patterns, as these cancer–testis antigens have been shown to be present as both nuclear and cytoplasmic proteins in cancer.

**Immunohistochemical analysis**

Immunohistochemical (IHC) analysis was performed on formalin-fixed paraffin-embedded tissues. Five micrometer TMA sections, and 4-μm whole sections, were deparaffinized, rehydrated, and treated in H2O2 to block the endogenous peroxidase activity. The sections were subjected to antigen retrieval by autoclaving for 15 minutes in 10 mmol/L citrate buffer, pH 6.0. The sections were incubated with the primary antibody for 1 hour at room temperature, followed by detection using DAKO Envision+ horseradish peroxidase mouse (or rabbit) detection system (DakoCytomation) and 3,3′-diaminobenzidine (DAB) as the chromogen. The slides were counterstained with hematoxylin and evaluated. Positive staining in any cancer cells, irrespective of percentage of positive cells or intensity, was regarded as positive.

Individual cancer–testis antibodies were used in the analysis of CT-X expression in resected tumors on TMA, and the expression frequency of individual CT-X antigens was determined. For biopsy specimens, a cancer–testis antibody cocktail consisting of six mouse monoclonal antibodies (against MAGEA, GAGE, NY-ESO-1, CT7, CT10, and CT45) was used. The biopsy specimens were not tested for SAGE (rabbit antibody) and NXF2 expression or for individual CT-X antigen expression.

### Table 1. Antibodies used in the present study

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Origin</th>
<th>Code</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAGEA</td>
<td>Mouse</td>
<td>6C1</td>
<td>1 μg/mL</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>NY-ESO-1</td>
<td>Mouse</td>
<td>E978</td>
<td>1 μg/mL</td>
<td>a</td>
</tr>
<tr>
<td>GAGE</td>
<td>Mouse</td>
<td>Clone 26</td>
<td>0.1 μg/mL</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CT7</td>
<td>Mouse</td>
<td>CT7-33</td>
<td>0.1 μg/mL</td>
<td>a</td>
</tr>
<tr>
<td>CT10</td>
<td>Mouse</td>
<td>LX-CT10-5</td>
<td>3 μg/mL</td>
<td>a</td>
</tr>
<tr>
<td>CT45</td>
<td>Mouse</td>
<td>LX-CT45-10</td>
<td>1:5,000 (ascites)</td>
<td>a</td>
</tr>
<tr>
<td>SAGE1</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>1.5 μg/mL</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>NXF2</td>
<td>Mouse</td>
<td>LX-NXF2-1</td>
<td>1:500 (ascites)</td>
<td>a</td>
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</tbody>
</table>

*aResearch antibodies produced in our laboratory.*
Results

The staining results are summarized in Table 2, and examples of positive immunostaining are illustrated in esophageal squamous cell carcinoma (Fig. 1) and adenocarcinoma (Fig. 2). Esophageal squamous cell carcinomas expressed cancer–testis antigens at higher frequencies than any other tumors. Positive staining for MAGEA, CT45, CT7, SAGE1, GAGE, NXF2, NY-ESO-1, and CT10 was observed in 57%, 38%, 33%, 33%, 29%, 29%, 19%, and 14% of esophageal squamous cell carcinoma, respectively (Fig. 1). These values were consistently higher than those observed in esophageal adenocarcinomas (18%, 24%, 5%, 18%, 16%, 13%, 10%, and 3%, respectively) and the differences in MAGEA and CT7 staining were statistically significant (P = 0.003 and 0.007, respectively). None of the 141 colorectal cancers express NY-ESO-1, CT7, CT10, or GAGE, but SAGE1 (0.7%) MAGEA (1.4%), CT45 (3.5%), and NXF2 (8.5%) staining was observed in a few cases. Carcinomas that expressed one cancer–testis antigen showed a tendency to simultaneously express multiple cancer–testis antigens (Table 3). This is particularly true in esophageal squamous cell carcinoma in which eight of 13 (62%) cancer–testis antigen–positive squamous cell carcinomas showed simultaneous expression of at least four cancer–testis antigens.

Correlations between cancer–testis expression and histopathologic features of the carcinomas were evaluated. The frequency of cancer–testis expression was not statistically different between gastric and esophageal adenocarcinomas. The histologic grade of the esophageal carcinoma (high-grade vs. low-grade) or the histologic type of the gastric adenocarcinoma (intestinal type vs. diffuse type) also did not affect the frequency of cancer–testis expression (P > 0.05 in all paired comparisons).

Twenty-two esophageal biopsy specimens with histologic diagnosis of squamous dysplasia or squamous cell carcinoma in situ were identified from 6 patients who were also diagnosed of invasive squamous cell carcinoma either subsequently or

<table>
<thead>
<tr>
<th>Table 2. Frequency of cancer–testis antigen expression in esophageal, gastric, and colon cancer</th>
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<tr>
<td></td>
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<tr>
<td>Esophagus</td>
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<tr>
<td>Adenocarcinoma</td>
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<tr>
<td>SCC</td>
</tr>
<tr>
<td>Stomach</td>
</tr>
<tr>
<td>Colon</td>
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Abbreviation: SCC, squamous cell carcinoma.

*P = 0.003 for MAGEA and P = 0.007 for CT7.

Figure 1. Expression of cancer–testis antigens in squamous cell carcinoma of the esophagus. This case expressed multiple cancer–testis antigens, including MAGE-A (A), NY-ESO-1 (B), CT7 (D), CT10 (E), CT45 (F), SAGE1 (G), and NXF2 (H and I), but was negative for GAGE (C). Note that MAGE-A and NY-ESO-1 showed nuclear and cytoplasmic staining. In comparison, CT7 showed only cytoplasmic staining in this case (see Fig. 2), and CT10, CT45, SAGE1, and NXF2 were nuclear proteins. The heterogeneous expression pattern of cancer–testis antigen in individual tumor was observed in this case in CT10 (E) and particularly in NXF2 (H), in which only a minority of the tumor cells were positive, as illustrated at higher magnification (I).
simultaneously. These noninvasive squamous lesions were analyzed with a cocktail of mouse monoclonal antibodies against six cancer–testis antigens (MAGEA, NY-ESO-1, GAGE, CT7, CT10, and CT45). Positive staining was observed in 18 of 22 biopsies (from 4 of 6 patients), including three of three biopsies diagnosed as low-grade dysplasia and 15 of 18 with high-grade dysplasia (Fig. 3). One patient had six sets of biopsies over a time period that spanned 23 months. Twenty-eight biopsy specimens were obtained during surveillance, including three diagnosed as low-grade dysplasia and 14 as high-grade dysplasia; the diagnosis of invasive squamous cell carcinoma was only made in the last set of biopsies. All 17 histologically dysplastic lesions showed diffuse, moderate to strong staining with the cancer–testis-cocktail antibodies, whereas the adjacent nondysplastic squamous epithelium were entirely negative for these markers (Fig. 3). The extent of cancer–testis-positive tumor cells was similar in all specimens and we did not observe any significant changes among these biopsies that would indicate a correlation between cancer–testis expression and tumor progression. This patient underwent esophageal mucosal resection and cryoablation during the 2-year surveillance period followed by esophagogastrectomy following the diagnosis of invasive carcinoma. The resection was performed 25 months after the first set of biopsies that showed cancer–testis antigen–positive dysplasia, and the specimen revealed a pT2N2 (stage IIIA) carcinoma.

Discussion

Although the expression of cancer–testis antigens in digestive tract malignancies has been studied at the RNA and protein levels, studies evaluating protein expression are mostly limited to MAGEA, NY-ESO-1, and GAGE (25, 27–30).

Table 3. Frequency of cancer–testis antigen coexpression in esophageal, gastric, and colon cancer (percentage of all cancer–testis-positive cases in parentheses)

<table>
<thead>
<tr>
<th></th>
<th>Express at least one cancer–testis antigen</th>
<th>Express at least two cancer–testis antigens</th>
<th>Express at least three cancer–testis antigens</th>
<th>Express at least four cancer–testis antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esophagus</td>
<td>26</td>
<td>21 (81%)</td>
<td>14 (54%)</td>
<td>12 (46%)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>13</td>
<td>11 (85%)</td>
<td>5 (38%)</td>
<td>4 (31%)</td>
</tr>
<tr>
<td>SCC</td>
<td>13</td>
<td>10 (77%)</td>
<td>9 (69%)</td>
<td>8 (62%)</td>
</tr>
<tr>
<td>Stomach</td>
<td>9</td>
<td>6 (67%)</td>
<td>5 (56%)</td>
<td>5 (56%)</td>
</tr>
<tr>
<td>Colon</td>
<td>15</td>
<td>3 (20%)</td>
<td>1 (7%)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Abbreviation: SCC, squamous cell carcinoma.

Figure 2. Expression of cancer–testis antigens in adenocarcinoma of the esophagus. This case expressed all eight cancer–testis antigens, i.e., MAGE-A (A), NY-ESO-1 (B), GAGE (C), CT7 (D), CT10 (F, but not in E, a different core of the same case), CT45 (G), SAGE1 (H), and NXF2 (I). CT10 was positive in less than 1% of the tumor cells, illustrating the heterogeneous expression of cancer–testis antigens. Also note that CT7 was present as both cytoplasmic and nuclear proteins in this case.
present study represents the most comprehensive analysis of cancer–testis protein expression in carcinomas of the esophagus, stomach, and colon. In esophageal squamous cell carcinoma, although 85% (26) or 90% (23) of these tumors were reported to be MAGEA mRNA-positive, IHC staining showed only 50% (antibody 57B; ref. 28) and 53% (27) MAGEA protein expression in two previous studies, similar to our finding of 58% in this study. This finding of more frequent cancer–testis mRNA expression than protein expression has also been observed in other types of cancer (4) and suggests that low-level cancer–testis mRNA expression may not translate to biologically significant protein expression. This observation is important in that it would suggest IHC staining of tumor as a better selection criterion than mRNA expression in identifying patients eligible for cancer–testis-based immunotherapy. Both phase III MAGEA3 trials on melanoma (DERMA) and on non–small cell lung cancer (MAGRIT) used MAGEA3 mRNA expression as the enrollment criterion, and clinical responses were preferentially observed in a subset of patients with a preferable gene signature (33). Given the heterogeneity of cancer–testis antigen expression in cancer, often expressed in a small percentage of tumor cells, it would be worthy to investigate whether the extent of MAGE protein expression in the tumor would also be another factor that might predict the likelihood of tumor response to MAGEA3 vaccine. In gastric cancer, Ogata and colleagues (29) reported MAGEA1 protein expression in 32.6% (44 of 135) of advanced gastric cancer, whereas Suzuki and colleagues (30) reported expression in only 9.8% (4 of 41) of gastric cancer of all stages. It is possible that at least part of this difference may indicate more frequent expression of MAGEA in late-stage gastric cancer, as 2 of 6 (33%) of T4 cancer in the series from Suzuki and colleagues were MAGEA1–positive compared with only 1 of 14 (7%) of the T1/2/3 tumors. On the other hand, it is difficult to compare these two studies, as Ogata and colleagues (30) only interpreted cases with cytoplasmic staining as MAGEA1–positive based on the debatable notion that MAGEA1 is a cytoplasmic protein (34), whereas Suzuki and colleagues (30) included both positive cytoplasmic and nuclear staining as MAGEA1–positive, which was the more commonly accepted criterion. [Of note, the 6C1 anti-MAGEA antibody that was used in these studies, including ours, in fact detects multiple MAGEA antigens, including MAGEA1, A2, A3, A4, A6, A10, and A12 proteins (31), and to interpret 6C1-positivity, either cytoplasmic and/or nuclear, as MAGEA1 expression (29, 30) is in fact misleading.] In our series, we found MAGEA expression in 14% (7 of 50) gastric cancer among all stages, indicating that the true frequency of MAGEA in gastric cancer is likely in the 10% to 15% range. In comparison with the upper gastrointestinal tract cancer, colon cancer showed by far the least frequent cancer–testis expression. This finding, previously well-documented at the mRNA level in MAGEA, GAGE (2), and NY-ESO-1 (7), is now confirmed at the protein level for these three and other cancer–testis antigens.

Our overall findings support and confirm several previously known characteristics of cancer–testis antigen expression in cancer. First, the frequency of cancer–testis expression is highly variable in tumor of different organ systems and primary colorectal carcinoma is “CT-poor” (4, 12). In contrast, esophageal squamous carcinoma is found to be a “CT-rich” cancer in our study. Second, the frequency of cancer–testis expression within the same organ system is dependent on the histologic type. We found that the adenocarcinomas of the esophagus express cancer–testis antigens less frequently than the
squamous cell carcinoma, similar to our previous observation in lung cancer (6). Third, cancer-testis antigens are often coexpressed as a cluster in cancer-testis-positive tumors, indicating that different cancer-testis antigens genes might be transcriptionally activated through a common mechanism, either by a possible master switch, for example, BORIS (35), or through epigenetic changes such as hypomethylation (36) that would simultaneously affect multiple cancer-testis genes. Fourth, the expression of cancer-testis antigens in individual tumor is often heterogeneous (4), consistent with the notion that cancer-testis expression is an epigenetic phenomenon rather than a clonal event. Finally, the same cancer-testis antigen can be expressed in different subcellular compartments, nuclear and/or cytoplasmic, of the tumor cells. This was previously described in MAGE, NY-ESO-1, and CT7, and patients with plasma cell myeloma with only cytoplasmic CT7 expression were found to have a better prognosis than patients with nuclear or combined nuclear and cytoplasmic CT7 expression (37). The control mechanism and biologic impact of this different subcellular localization of cancer-testis antigens, however, are unclear.

Other findings, however, were different from those previously reported in other types of malignancy by us and by others. First, cancer-testis antigens have been shown to be more frequently expressed in high-grade carcinomas in several tumor types, including bladder cancer (19, 20) and breast cancer (22, 32). In this study, however, no such difference was found, for example, between the more differentiated intestinal-type gastric adenocarcinoma and the high-grade diffusive-type carcinoma, or between the high-grade and low-grade esophageal carcinoma. This lack of correlation of cancer-testis expression to histologic grade is similar to that reported by quantitative analysis of the cancer-testis mRNA in gastric carcinoma (25). Second, cancer-testis antigens have been implicated as a marker for tumor progression, expressed more frequently in late stage of malignancy, for example, in metastatic melanoma versus primary melanoma (2) and in late-stage urothelial carcinoma (19). Our finding that cancer-testis antigens are expressed in squamous cell carcinoma in situ and in dysplastic lesions contradicts this notion. In this regard, it is of interest that Bhutani and colleagues (38) reported the detection of MAGEA mRNA in the bronchial brushing of heavy smokers in the absence of lung cancer, leading to the proposal that MAGEA expression is initiated in the bronchial epithelium following exposure to tobacco carcinogenic insult even before malignant transformation. Because squamous cell carcinoma is the main type of smoking-related carcinoma in the major airways, it is possible that squamous cell carcinoma might represent a special tumor type in which the cancer-testis gene activation is often an early event. We have recently analyzed squamous cell carcinoma and dysplasia in the head and neck region, and cancer-testis antigens were similarly observed in a subset of squamous dysplasia (39), further supporting this possibility. This would suggest that cancer-testis antigens could be useful markers of early malignant transformation in squamous cell carcinogenesis, and it would be worthwhile to explore this possibility in a prospective study of dysplastic squamous lesions with long-term follow-up.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: Y.-T. Chen, R.K. Yantiss
Development of methodology: R.K. Yantiss
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.-T. Chen, N.C. Panarelli, K.C. Piotti
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.-T. Chen, N.C. Panarelli, R.K. Yantiss
Writing, review, and/or revision of the manuscript: Y.-T. Chen, N.C. Panarelli, R.K. Yantiss
Study supervision: Y.-T. Chen

Acknowledgments

The authors are grateful for the long-term support of the late Dr. Lloyd J. Old to our work in the definition and characterization of cancer-testis antigens over the past two decades. This work would not have been possible without his mentorship and support to Y.-T. Chen.

Grant Support

This study was supported by Ludwig Institute for Cancer Research Funds (to Y.-T. Chen)

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Received August 13, 2013; revised October 11, 2013; accepted November 5, 2013; published OnlineFirst November 11, 2013.

References


Cancer Immunology Research

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Cancer Immunol Res Published OnlineFirst November 11, 2013.

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